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X-Inactivation Patterns in Female Leber's Hereditary Optic Neuropathy Patients Do Not Support a Strong X-linked Determinant

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Leber's hereditary optic neuropathy (LHON) accounts for about 3% of the cases of blindness in young adult males. The underlying mitochondrial pathogenesis of LHON has been well studied, with specific mitochondrial DNA (mtDNA) mutations of structural genes described and well characterized. However, enigmatic aspects of the disease are not explained by mutation data, such as the higher proportion of affected males, the later onset of the disease in females, and the presence of unaffected individuals with a high proportion of mutant mtDNA. A hypothesis which has been put forward to explain the unusual disease expression is a dual model of mtDNA and X-linked nuclear gene inheritance. If a nuclear X-linked modifier gene influences the expression of the mitochondrial-linked mutant gene then the affected females should be either homozygous for the nuclear determinant, or if heterozygous, lyonization should favor the mutant X. In order to determine if an X-linked gene predisposes to LHON phenotype we studied X-inactivation patterns in 35 females with known mtDNA mutations from 10 LHON pedigrees. Our results do not support a strong X-linked determinant in LHON cause: 2 of the 10 (20%) manifesting carriers showed skewing of X-inactivation, as did 3 of the 25 (12%) nonmanifesting carriers.

KEY WORDS: **Leber's hereditary optic neuropathy, X-inactivation, X-linked inheritance, mitochondrial genetics**

INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is one of the common causes of blindness in young men. The onset of the disease is usually in the second to third decade with a centro-caecal scotoma and optic atrophy that lead to acute or subacute bilateral visual loss. The decreased visual acuity is often preceded by peripapillary microangiopathy. LHON affects almost exclusively the optic nerve, although there have been reports of associated electrocardiographic changes and various neurological signs [reviewed by Harding and Sweeney, 1994].

LHON is maternally inherited. The molecular cause of the disease was advanced considerably in 1988 by Wallace et al. [Wallace et al., 1988; Singh et al., 1989] with the discovery of a mitochondrial (mtDNA) missense mutation at nucleotide 11778 in the mitochondrial respiratory chain complex I subunit. Since then, at least 5 other causative missense mutations have been described in different subunits of complex I, supporting genetic heterogeneity in LHON [Huoponen et al., 1991; Howell et al., 1991; Wallace et al., 1992a; Mackey and Howell, 1992]. Heteroplasmy dictates that patients can show a different proportion of mutant and normal mitochondria, a characteristic that also adds to clinical variability.

However, genetic heterogeneity and heteroplasmy alone do not explain peculiar aspects of LHON's inheritance pattern. For example, the predominance of affected males (50% of mutation-carrying males, but only 20% of females in LHON maternal lineages are affected [Seedorf, 1985; Nikoskelainen et al., 1987]), the later onset of the disease in females (6–30 years later in comparison with males [Nikoskelainen et al., 1983]), and

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the presence of unaffected males and females with high proportions of mutant mtDNA [Holt et al., 1989] all suggest the possibility of a dual mitochondrial and X-chromosome-linked nuclear gene inheritance model in the etiopathogenesis of the disease [Wallace, 1987].

To test this hypothesis, Chen et al. [1989] performed multipoint analysis using 15 X-chromosome markers in 3 large Tasmanian families, and showed data suggesting the exclusion of the involvement of an X-linked gene. To the contrary, Vilkki et al. [1991] obtained a maximum lod score of 2.48 at a recombination fraction (Θ) of 0 for the anonymous marker DXS7, a finding which placed a hypothetical locus for the liability to develop optic atrophy in the proximal region of the short arm of the X chromosome. Simultaneously, Bu and Rotter [1991] used segregation analysis of over 1,200 individuals from published pedigrees to confirm the 2 locus nuclear and mitochondrial mode of inheritance. Using the same approach, similar results were attained in large Japanese pedigrees [Nakamura et al., 1993b]. However, in 1992 a study of 129 patients in one Italian and 12 British families with LHON excluded linkage at the DXS7 locus [Sweeney et al., 1992]. Moreover Vilkki's group used revised pedigrees, liability classes, and separation of the families according to the associated mtDNA mutation and consequently reviewed their previous results and were not able to find any evidence of linkage to any X-chromosomal locus [Juvonen et al., 1993]. If an X-linked recessive LHON modifier locus does indeed exist at a high incidence in the general population, then it would be expected that many LHON affected females are in fact "manifesting carriers" of the X-linked locus. Nearly all manifesting carriers of X-linked recessive disorders show "skewed X-inactivation": the normal X chromosome is preferentially inactivated, leading to expression of the disease as a heterozygote [Conley et al., 1990; Goodship et al., 1988, 1991; Puck et al., 1987; Pegoraro et al., 1994]. If, in fact, an X-linked modifier locus exists, many or most female LHON patients should show patterns of X-inactivation that are more "skewed" than in nonmanifesting females. To test this hypothesis, we determined the X-inactivation patterns in 10 females affected with LHON and 25 females carrying the mitochondrial mutation but having normal visual acuity. Our results on X-inactivation patterns in female carriers showed no association of skewed inactivation with manifestation of visual loss.

MATERIALS AND METHODS

Diagnosis and Clinical Manifestations in Females From LHON Pedigrees

We studied 35 female patients from LHON pedigrees found to have primary mtDNA mutations. All the patients included in this study met the following criteria: female sex, presence of primary mtDNA mutation (11778/ND4, 3460/ND1, 14484/ND6), and availability of clinical records. Patients from families 1–6 were evaluated at the Institute of Clinical Neurology of the University of Bologna, and patients from families 7–10 at "Besta" Neurological Institute in Milano. Affection status was determined by the presence of optic atrophy and visual field defects. Automated static perimetry was done to detect both central and peripheral scotomas. Fluorescein angiography was performed to demonstrate peripapillary microangiopathy.

DNA Analyses

Blood DNA. Blood was collected in EDTA tubes. DNA was isolated from peripheral blood as previously described [Maniatis et al., 1982].

mtDNA studies. Identification of the primary mtDNA mutations 11778/ND4, 3460/ND1, and 14484/ND6 were done as previously described [Cortelli et al., 1991; Huoponen et al., 1991; Johns et al., 1993]. The presence of 11778/ND4 mutation was detected by the combined use of SfaI (loss of site) and MaeIII (new site) restriction endonucleases. The 3460/ND1 mutation was identified by loss of a AhaII/AcyI restriction site, and 14484/ND6 mutations by loss of a Sau3AI restriction site. Associated, secondary mtDNA mutations at 15257/cytb, 15812/cytb, 13708/ND5, 3396/ND1, 4216/ND1, 4917/ND2, 5244/ND2, and 7444/COI were also screened according to methods described elsewhere [Johns and Berman, 1991; Brown et al., 1992a,b; Johns et al., 1992]. Heteroplasmy was determined comparing the intensity of the band of the normal and mutated mtDNA with an internal marker used as control.

X-inactivation studies. Polymerase chain reaction (PCR) of androgen receptor was done as previously described [Allen et al., 1992; Pegoraro et al., 1994]. Briefly, the polymorphic androgen receptor CAG repeat sequence in the 5′ coding region of the gene was amplified from genomic DNA to define the 2 X chromosomes of each female, and the methylation status of each allele was determined using the methylation-sensitive restriction enzymes HpaII/CfoI. All PCR products were electrophoresed on an ABI 373A automated sequencer. Peak heights and areas were analyzed using GeneScan software (Applied Biosystems). Equal reduction of both alleles after digestion indicates random X-inactivation, where the preferential inactivation of one allele appeared as complete digestion of one of the androgen receptor alleles. Semiquantitation of X-inactivation was done as described previously [Pegoraro et al., 1994].

A ratio of 25%:75% between the 2 alleles of the androgen receptor was used as the cut-off point between the random X-inactivation and the skewed X-inactivation patients [Pegoraro et al., 1995].

Statistical Analysis

Analysis of variance (ANOVA) single factor was used to compare the distribution of the values of X-inactivation in the females manifesting LHON and the females harboring the mtDNA mutation without visual impairment.

RESULTS

mtDNA Studies

Thirty-five females from 10 LHON pedigrees were studied. Families 3–10 have the primary 11778/ND4 mutation, and families 1 and 2 the primary 3460/ND1 mutation (Table I). (Clinical and molecular data on family 6 were published previously [Cortelli et al.,

TABLE I. Clinical and Molecular Data on 35 Females From LHON Pedigrees

Patient no.	Homoplasmy	% of active X		Clinical symptoms
		Paternal	Maternal	
Family 1				
3460/ND1				
374A	Yes	15	85	Asymptomatic
374B	Yes	80	20	Asymptomatic
374C	~60% Mutant	Homozygous		Asymptomatic
374E	Yes	60[a]	40[a]	Optic atrophy
Family 2				
3460/ND1				
375A	~10% Mutant	60	40	Asymptomatic
375B	~30% Mutant	80	20	Optic atrophy Anorexia
375C	~10% Mutant	45	55	Asymptomatic
375D	No mutant	60[a]	40[a]	Asymptomatic
Family 3				
11778/ND4				
376A	Yes	15	85	Optic atrophy Epilepsy
376B	Yes	10[a]	90[a]	Asymptomatic
Family 4				
11778/ND4[b]				
378A	Yes	Homozygous		Optic atrophy
378E	Yes	Homozygous		Asymptomatic
378F	Yes	40	60	Asymptomatic
378G	Yes	45	55	Asymptomatic
378H	Yes	55	45	Asymptomatic
378I	Yes	70	30	Asymptomatic
378L	Yes	35	65	Asymptomatic
Family 5				
11778/ND4[c]				
P1	Yes	75[a]	25[a]	Optic atrophy
Family 6				
778/ND4				
379C	~75–95% Mutant	Homozygous		Asymptomatic
379D	Yes	Homozygous		Asymptomatic
379E	Yes	Homozygous		Asymptomatic
379G	~75–95% Mutant	Homozygous		Asymptomatic
379L	~75–95% Mutant	50	50	Asymptomatic
379M	~50–70% Mutant	60[a]	40[a]	Asymptomatic
379N	Yes	50	50	Asymptomatic
379Q	~75–95% Mutant	50	50	Asymptomatic
379R	Yes	45	55	Asymptomatic
Family 7				
11778/ND4				
278	Yes	55[a]	45[a]	Optic atrophy
Family 8				
11778/ND4				
642	Yes	60[a]	40[a]	Asymptomatic
639	Yes	60[a]	40[a]	Optic atrophy
Family 9				
11778/ND4[d]				
498	Yes	65[a]	35[a]	Asymptomatic
502	Yes	Homozygous		Optic atrophy
144	Yes	60[a]	40[a]	Optic atrophy
Family 10				
11778/ND4				
553	Yes	70[a]	30[a]	Optic atrophy
556	Yes	55[a]	45[a]	Asymptomatic

[a]Parental origin of each allele was not determined.
[b]Family 4 also has the mutations 13708/ND5 and 4216/ND2.
[c]Family 5 also has the mutations 13708/ND5 and 4216/ND2.
[d]Family 9 also has the mutation 15257/cyt b.

1991]). Families 4 and 5 also have the associated 13708/ND5 and 4216/ND2 mtDNA mutations, and family 9 an additional mutation at 15257 of the cytochrome b subunit.

Seven of 10 studied families were homoplasmic for the primary mtDNA mutation (families 3–5, 7–10). Females in family 6 had a high degree of mutant mtDNA, from 100% to 50–70%, whereas in the females of family 2 a low degree of mutant mtDNA was detected: from barely visible mutant mtDNA to 30% of mutant. Only one female in family 1 had 60% of heteroplasmy (Table I). All the associated mtDNA mutations were virtually homoplasmic.

Clinical Diagnosis of LHON

Ten of these 35 women were affected with LHON, having both optic atrophy and central scotoma except one (375B) with arcuate field defect. The age-of-onset ranged between 9 and 40 years. Twenty-five of the 35 patients were asymptomatic. Their ages ranged between 19 and 59 years. In 20 asymptomatic patients, fluorescein angiography was performed and 18 of these showed peripapillary microangiopathy.

X-Inactivation Studies

Genomic DNA from each of the 35 females in this study was used to determine the ratio of active and inactive X chromosomes. Among the females affected with LHON, 2 of 8 (25%) showed a skewed pattern of X-inactivation in their peripheral blood DNA with a ratio between the active and inactive X of 20%:80% and 15%:85%; 6 of 8 (75%) a ratio between 55%:45% to 25%:75% (Table I). According to previously used criteria [Pegoraro et al., 1995], only 2 patients in this group were scored as having skewed X-inactivation (Fig. 1). For these 2 patients (375B and 376A, Table I) it was possible to determine the parental origin of the preferentially active X by comparing the peaks of the androgen receptor in the patient with those of the parents. In both cases the preferentially active X was paternal in origin. Patient 376A who is affected with LHON represents a peculiar case. She is the only child of consanguineous parents. Both parents (376C and 376B) were homoplasmic for the same 11778/ND4 mutation inheriting the mtDNA mutation through maternal lineages. Patient 376A uses the paternal X in 85% of her peripheral blood cells and the maternal X in the remaining 15% (Fig. 2). In the group of patients carrying the mtDNA mutation, but with normal visual acuity, 3 of 19 (16%) were skewed and 16 of 19 (84%) had a random pattern of X-inactivation (Table I, Fig. 2).

All the females affected with LHON had lost central vision but 2 spontaneously recovered 20/20 visual acuity after a month (375B) and 8 years (378A) and in no case it was possible to correlate the extent of X-inactivation with the severity of the visual impairment. Equally in the asymptomatic females group there was no clear correlation between the extent of X-inactivation and the abnormalities detected by fluorescein angiography at the fundus (Table I).

Twenty percent (2 of 10) of the affected females and 32% (6 of 19) of the asymptomatic females were homozygous for the androgen receptor. The discrepancy between this high homozygosity ratio (26%) and that previously reported [10%, Allen et al., 1992] may be related to the large number of sibs used in this study or may reflect the allele distribution in the Italian population study.

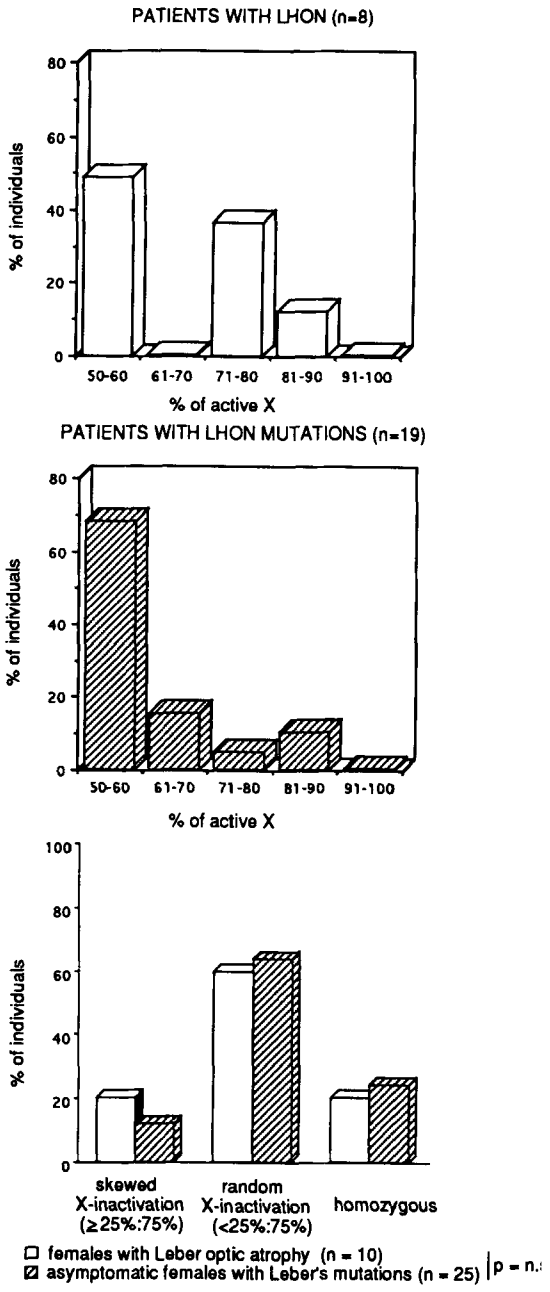

Fig. 1. Comparison of X-inactivation patterns in peripheral blood DNA in 10 females affected with LHON and 25 unaffected females with LHON mtDNA mutation. Shown is the distribution of the extent of X-inactivation in the patients affected with LHON (**top**) and in the asymptomatic LHON mutation-carrying females (**center**). Ratios of inactivation were determined as described in Materials and Methods. The percentage of skewed and random X-inactivation patients in the 2 groups studied is shown (**bottom**). All values were rounded to the nearest 5.

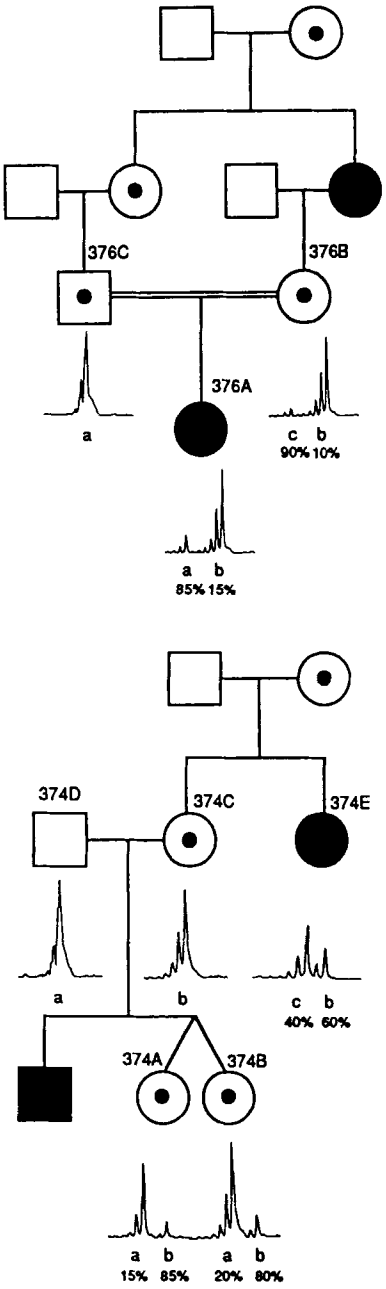

Fig. 2. Inheritance of mtDNA mutation and X-inactivation patterns in 2 families segregating LHON. Pedigree for patient 376A (top) and pedigree for patient 374E (bottom). Symbols with dots indicate unaffected patients homoplasmic for the mtDNA mutation, filled symbols indicate affected homoplasmic patients. Automated sequencer traces for the androgen receptor alleles are shown under each symbol. Also provided are the percentage of cells showing each X chromosome active. **Top:** Patient 376A is homoplasmic for the 11778/ND4 mtDNA mutation and is affected with LHON. Both her parents (376B and 376C), who are cousins, are homoplasmic for the 11778/ND4 mtDNA mutation and are asymptomatic. The mtDNA mutation is transmitted through maternal lineages. X-inactivation study in this family shows that 376A is using the paternal X in 85% of her peripheral blood cells. Father (376C) and daughter (376A) share the same X, but clinical manifestation of LHON is present only in the daughter (376A). 376B is asymptomatic, and has skewed X-inactivation in her peripheral blood. Her preferentially inactive X (allele "b") is also preferentially inactive in her daughter (376A) (allele "b"). This family argues against a strict X-linked determinant in LHON. The unaffected father (376C) probably does not carry the hypothetical X-linked modifier locus as he is unaffected. Furthermore, the modifier locus probably does not come from the mother as both the unaffected mother (376B) and affected daughter (376A) show similar X-inactivation patterns. **Bottom:** Patient 374E is homoplasmic for the 3460/ND1 mtDNA mutation and is affected with LHON. The mtDNA mutation is transmitted through maternal lineages to her twin nieces. X-inactivation study in this family shows that 374E has a random pattern of X-inactivation using roughly equal amounts of the paternal and maternal X in her blood cells, where the twin nieces (374A and 374B), who have skewed X-inactivation, are asymptomatic. This family shows that the extent of X-inactivation does not correlate with clinical phenotype in LHON.

Statistical Analysis

ANOVA single factor was used to compare the distribution of the values of X-inactivation between the group of females affected with LHON and the females with only the mtDNA mutation. The 2 groups did not show a statistically significant difference ($P > 0.3$). In addition, no statistically significant difference was seen when only the homoplasmic females in the 2 groups (average of the values of X-inactivation were 64% ± 12% in the LHON-affected females vs. 65% ± 11% in the asymptomatic LHON females) were studied ($P > 0.8$).

DISCUSSION

The primary pathogenic mtDNA mutations in LHON have been well studied [Wallace et al., 1988; Singh et al., 1989]; however, the dual inheritance model of a nuclear X-linked modifier gene is still a topic of debate. We studied X-inactivation patterns in 35 females from LHON pedigrees as an alternative approach to addressing this issue. All of the females studied had the 11778/ND4 or the 3460/ND1 mtDNA mutation that has been previously shown to be pathogenic in LHON [Wallace et al., 1988; Singh et al., 1989; Huoponen et al., 1991; Howell et al., 1991].

The relative proportion of mutant mtDNA can modulate the degree of severity of the clinical symptoms [Isashiki and Nakagawa, 1991; Zhu et al., 1992]. In our cohort of females, 9 of 10 (90%) affected patients were homoplasmic for the mutation, and 9 of 25 (36%) of the unaffected females were also homoplasmic. Consistent with other reports, homoplasmy alone does not explain the clinical phenotype in our patient population: We show that in the same family, homoplasmy is present in both affected and unaffected females [families 3, 4, 8–10; Nakamura et al., 1993a]. In only one of the 2 families in which heteroplasmy was present is it possible to postulate a threshold effect: The affected female (375B) had the higher percentage of mutant mtDNA (~30%) in comparison with the unaffected relatives (375A, 375C, 375D) (~10%).

Synergism of multiple mtDNA mutations cannot be ruled out as a modulating factor in clinical phenotype expression, but again it does not seem to explain the in-

trafamilial variability [Gerbitz et al., 1992]. Families 4 and 5 had both the 13708/ND5 and secondary 4216/ND2 mtDNA mutation, and family 9 had the 15257/cyt associated mtDNA mutation, but in all 3 families there were affected and unaffected females with a similar degree of homoplasmy.

To attempt and garner support for the existence of an X-linked modifier locus we studied X-inactivation patterns in affected and unaffected females. Segregation analysis methods [Bu and Rotter, 1991; Nakamura et al., 1993b] in extended pedigrees have suggested a recessive inheritance pattern for a hypothetical X-linked modifier locus. If an X-linked recessive modifier gene exists, and this locus is subject to X-inactivation as with most X-linked genes, then many if not most affected females would be expected to show skewed X-inactivation, as has been shown for most other X-linked recessive disorders [Conley et al., 1990; Goodship et al., 1988, 1991; Puck et al., 1987; Pegoraro et al., 1994]. For this reason we assessed X-inactivation in 35 females from LHON pedigrees to verify if the extent of X-inactivation correlated the molecular findings with the clinical manifestation of LHON. The patients were scored as affected with LHON if optic nerve atrophy and central scotoma were present. Ten of the 35 patients met these criteria. In 25 females the mtDNA mutation was present in the absence of clinical signs of visual impairment. Using the value of 25%:75% as the cut-off [Pegoraro et al., 1995], we found only 2 of the 8 (25%) informative patients with LHON to have skewed X-inactivation patterns. In the patients without clinical signs of LHON, 12% showed skewed X-inactivation. Our data show that there is not any statistically significant difference between subsets of patients, and that clinical manifestation of LHON does not appear to depend on the extent of X-inactivation. The percentage of patients with skewed X-inactivation in both patient groups is not substantially different from percentages observed in normal females [Harris et al., 1992; Pegoraro et al., 1994]. Furthermore, if we compare the subsets of patients having values of X-inactivation between 60% to 83%, values that have been suggested by Bu and Rotter [1992] to be the disease threshold for a heterozygous female, we found that only 36% of the females affected with LHON fit this category. Moreover, 21% of females with the LHON mutation but no visual impairment also fit the range suggested by Bu and Rotter [1992].

Using segregation analysis it has been calculated that about 40% of LHON affected females might be homozygous for the X-linked determinant. Including also homozygosity ratio calculation in our affected females group, 60% of them would be expected to show skewed X-inactivation according to this model; however, we observed only 25% skewing. Again, similar assumptions regarding homozygosity do not fit the data in the clinically normal mutation-carrying females in our study.

Despite the lack of data supporting a strong X-linked determinant in LHON, other possibilities have to be considered before rejecting the hypothesis of a nuclear involvement in the disease. LHON is a tissue-specific disease and the optic nerve is the target tissue in the pathogenetic process. X-inactivation patterns in lymphocyte DNA do not necessarily reflect patterns present in the optic nerve. As we are unable to assay X-inactivation patterns in the optic nerve, it remains possible that affected females show skewed X-inactivation patterns in the optic nerve and not in peripheral blood cells. Indeed, it was recently showed that the degree of heteroplasmy of the optic nerve does not reflect the pattern of the peripheral blood DNA in a single autopsy case studied [Sadun et al., 1994]. On the other hand a recent study comparing peripheral blood DNA to mucosal epithelial cell DNA showed very consistent patterns of X-inactivation in the majority of females [Pegoraro et al., unpublished]. A larger series of autopsy cases should probably be done before drawing firm conclusions. Reduced penetrance and nuclear genes that escape X-inactivation are other possible modulating factors in the final expression of LHON phenotype.

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